# PURIFICATION AND PROPERTIES OF A CHROMOSOMAL β-LACTAMASE FROM *Klebsiella oxytoca*

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A β-lactamase was purified from *Klebsiella oxytoca* strain GN10650. The enzyme was chromosomally-mediated and gave a single protein band on polyacrylamide gel electrophoresis. Its pI was 5.34 and its MW was approximately 27,000. The optimal pH and temperature were about 7.0 and 50°C, respectively. The specific activity of the enzyme was 1,207 units per mg of protein for hydrolysis of penicillins and cephalosporins, including cefuroxime, cefotaxime, and aztreonam. The enzyme activity was inhibited by *p*-chloromercuribenzoate, iodine, ferrous ion, and by clavulanic acid. Rabbit antibodies raised against the purified *K. oxytoca* enzyme showed no cross-reactivity in neutralization tests with β-lactamases produced by other species of Gram-negative bacteria.

Indole-positive strains of *Klebsiella* are grouped as a separate species, *Klebsiella oxytoca*[1]. Considerable difference has been reported between *Klebsiella pneumoniae* and *K. oxytoca* with respect to pathogenicity, antibiotic susceptibility and occurence of drug resistance plasmids[2~5].

Most β-lactam antibiotics show relatively poor activity against *K. oxytoca*[5], perhaps because numerous strains produce a semi-inducible or constitutive β-lactamase, which is considered to be species specific. However, there has been no direct report on the activity of this enzyme except that of SPITTON *et al.*[6]. We report here the purification and biochemical properties of a β-lactamase produced by *K. oxytoca* strain GN10650.

## Materials and Methods

### Antibiotics

Benzylpenicillin, ampicillin, carbenicillin, cloxacillin, cephaloridine, cephalothin, and cefazolin were commercially available materials. We received the following compounds as gifts from manufacturers: cefuroxime (Glaxo Group Research Ltd.), cefotaxime (Hoechst Japan Ltd.), ceftizoxime (Fujisawa Pharm. Co., Ltd.), cefmetazole (Sankyo Co., Ltd.), cefoxitin and imipenem (Merck Banyu Co., Ltd.), cefoperazone (Toyama Chemical Co., Ltd.), latamoxef (Shionogi & Co., Ltd.), cefsulodin (Takeda Chemical Industries, Ltd.), aztreonam (Eisai Co., Ltd.) and clavulanic acid (Beecham Yakuhin Co., Ltd.).

### Bacterial Strains

A total of 33 strains of *K. oxytoca* were isolated from clinical material at different hospitals in Japan and maintained in cooked meat medium (Eiken Chemical Co., Tokyo, Japan).

### Antibiotic Resistance

MICs of various β-lactam antibiotics were determined on Sensitivity disk agar N (Nissui, Tokyo, Japan). One loopful (*ca.* 5 μl) of diluted culture (*ca.* $5 \times 10^6$ cfu/ml) in Sensitivity test broth (Nissui, Tokyo, Japan) was inoculated onto the assay media containing serial 2-fold dilutions of drugs. MICs were estimated after incubation at 37°C for 18 hours.

### Culture and Harvesting of Microorganisms

Medium B broth[7] in a 5-liter flask was warmed to 37°C and inoculated with 100 ml of an overnight

culture, then incubated on an rotary shaker at 37°C. After 4 hours, the bacteria were harvested by centrifugation and washed once with 0.05 M sodium phosphate buffer, pH 7.0. Cells were resuspended in 200 ml of 1 mM phosphate buffer, pH 7.0 and treated for 4 minutes in an ultrasonic disintegrator (80W, 25 kHz). The sonicate was then centrifuged at 10,000 × $g$ for 20 minutes at 5°C and cell debris and the supernatant was retained as the crude enzyme preparation.

Determination of protein concentration was carried out by the LOWRY method[8] with bovine serum albumin as a standard.

Purification of β-Lactamase

For the preparation of crude enzymes, we followed the method described previously[9]. The β-lactamases were purified by absorption and elution on a DEAE-cellulose column (2.5 by 42 cm) and gel filtration on a Sephadex G-200 column (2 by 62 cm). The active fractions were pooled and applied again to another column of DEAE-cellulose and on a Sephadex G-200 column.

Isoelectric Focusing

Electrofocusing was performed on a sucrose ampholyte gradient (pH 3.5 to 10.0; LKB Produkter AB, Stockholm, Sweden). The experiment was carried out below 5°C for 48 hours in an Ampholine electrofocusing column, model LKB8100 with a final potential of 300 V. The contents of the column were separated into 3 ml fractions, and each fraction was assayed for its enzyme activity and pH.

Determination of MW

The MW was estimated by gel filtration through Sephadex G-200 with 0.05 M Tris-HCl - 0.1 M NaCl buffer, pH 8.0 as the eluent as described by ANDREWS[10] and by the SDS-PAGE method of LAEMMLI and FARVE[11]. In the latter procedure, purified enzyme and marker proteins were treated with 10% sodium dodecyl sulfate - 3% 2-mercaptoethanol at 100°C for 2 minutes and then subjected to electrophoresis for 6 hours at 0°C in a 12% gel with a current of 25 mA. The marker proteins used (LMW kit, Pharmacia) and their MW were: phosphorylase b (94 k), albumin (67 k), ovalbumin (45 k), carbonic anhydrase (30 K), trypsin inhibitor (20.1 k), and lactoalbumin (14.4 k).

Assay of β-Lactamase and Inhibition Study

The β-lactamase activity was measured by the spectrophotometric method described previously[12] and by a modification of the microiodometric method[13]. The Michaelis constant ($Km$) and the maximum rate of hydrolysis (Vmax) were determined from Lineweaver-Burk plots. The dissociation constants ($Ki$) of enzyme-inhibitor complexes were determined from Dixon plots, using cephaloridine as a substrate. One unit of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at 30°C in 50 mM phosphate buffer (pH 7.0).

Immunological Methods

Rabbit antibodies were produced against the purified enzyme preparation from strain GN10650. The first injection, 1 mg enzyme protein in 0.5 ml saline, was administered subcutaneously. The second subcutaneous injection, 1 mg protein in 0.5 ml saline, was emulsified with 0.5 ml complete FREUND's adjuvant (Difco). After 3 weeks a booster injection containing 0.75 mg protein, was administered in the marginal ear vein. Antisera were collected about 2 weeks after the last injection.

β-Lactamase preparations were analyzed by the micro double-immunodiffusion technique reported by SAWAI *et al.*[14].

The effect of the antibodies on β-lactamase activity was determined by a neutralization test of the hydrolyzing activity of the enzyme. The enzyme solution (about 0.6 unit) was incubated with various amounts of antibody in 0.2 ml of 0.1 M phosphate buffer, pH 7.0 at 37°C for 20 minutes and then left at 5°C for 18 hours. Subsequently, the enzyme activity was determined by spectrophotometric assay.

## Results

### Ampicillin-susceptibility and Substrate Profile of β-Lactamase

Twenty four of the *K. oxytoca* isolates were resistant to ampicillin (MIC > 12.5 μg/ml) and 9 strains

were further resistant to several antibiotics such as cephaloridine, cefamandole and cefotaxime.

Five strains (GN10553, GN10639, GN10641, GN10647 and GN10650) highly resistant (MIC) to ampicillin were examined in details. Ampicillin-resistance was not transferable by conjugation or by transformation using total DNA. Their $\beta$-lactamase specific activities against cephaloridine as a substrate were 1.21 to 4.09 U/mg protein. The enzymes hydrolyzed not only ampicillin, benzylpenicillin, carbenicillin but also cephaloridine. *K. oxytoca* GN10650 had the highest enzyme activity and was used as a source of $\beta$-lactamase for purification.

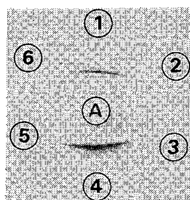
## Purification of $\beta$-Lactamase

The purification procedure of the enzyme from *K. oxytoca* GN10650 is summarized in Table 1. The enzyme was purified about 670-fold from the crude extract and gave a single protein band on SDS-polyacrylamide slab gel electrophoresis (see Fig. 2). Agar gel immunoelectrophoresis using the anti-GN10650 antibody gave a single precipitin arc, showing that the antibodies contained a single component reacting with crude enzyme. Moreover, the precipitin arc for the antibody and the purified enzyme preparation was symmetrical to that of the crude enzyme (Fig. 1).

## Physico-chemical Properties of the Purified Enzyme

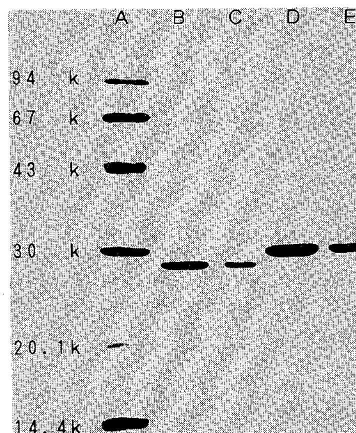
The approximate MW of the purified enzyme was 27,000, as estimated by gel electrophoresis (Fig. 2). The isoelectric point (pI) was 5.3. We could not detect neutral sugar in the purified enzyme by the

Fig. 1. Immunodiffusion analysis of $\beta$-lactamase from *Klebsiella oxytoca*.




The antiserum against $\beta$-lactamase of *K. oxytoca* GN10650 was placed in the central well (A). The outer wells contained the antigen; crude enzyme (well No. 1) and purified enzyme (well No. 4) from *K. oxytoca* GN10650, crude enzyme from *K. pneumoniae* GN69 (well Nos. 2 and 5) and type 1 penicillinase of Rms212 (well Nos. 3 and 6).

Fig. 2. The MW of purified $\beta$-lactamase from *Klebsiella oxytoca* GN10650 by the SDS-PAGE.




Lane A: Marker proteins, lane B: purified $\beta$-lactamase from *K. oxytoca* GN10650 (7 $\mu$g), lane C: two times dilution of lane B, lane D: purified $\beta$-lactamase from *Proteus vulgaris* GN7919 (7 $\mu$g), lane E: two times dilution of lane D.

Table 1. Summary of the purification of the $\beta$-lactamase from *Klebsiella oxytoca* GN10650.

| Stage | Procedure | Recovered activity (units) | Specific activity (units/mg protein) | Purification (-fold) | Recovery (%) |
|---|---|---|---|---|---|
| 1 | Ultrasonic disintegration followed by streptomycin treatment | 11,414.2 | 1.79 | 1 | 100 |
| 2 | Chromatography on DEAE-cellulose | 6,854.6 | 174.1 | 97 | 60 |
| 3 | Gel filtration on Sephadex G-200 | 4,724.0 | 358.8 | 200 | 41 |
| 4 | Chromatography on DEAE-cellulose | 3,036.8 | 654.6 | 357 | 277 |
| 5 | Gel filtration on Sephadex G-200 | 1,629.5 | 1,207.0 | 674 | 14 |

Table 2. Kinetics of hydrolysis of various β-lactam antibiotics by the β-lactamase from *Klebsiella oxytoca* GN10650.

| Substrate | *Km* (μM) | *Ki* (μM) | Vmax (relative to cephaloridine) | Substrate | *Km* (μM) | *Ki* (μM) | Vmax (relative to cephaloridine) |
|---|---|---|---|---|---|---|---|
| Cephaloridine | 89.3 | | 100 | Cefbuperazone | | 60.1 | <0.1 |
| Cephalothin | 69.4 | | 117.3 | Latamoxef | | — | <0.1 |
| Cephalexin | 289.7 | | 8.5 | Cefsulodin | 690.5 | | 11.8 |
| Cefamandole | 74.9 | | 127.7 | Benzylpenicillin | 98.0 | | 206.1 |
| Cefoperazone | 1.1 | | 0.3 | Ampicillin | 122.1 | | 183.2 |
| Cefuroxime | 85.2 | | 22.6 | Carbenicillin | 105.5 | | 27.8 |
| Cefotaxime | 366.4 | | 5.3 | Cloxacillin | 143.3 | | 17.4 |
| Ceftizoxime | 42.2 | | <0.1 | Imipenem | | 33.7 | <0.1 |
| Cefmetazole | | N | <0.1 | Aztreonam | 137.0 | | 12.0 |
| Cefoxitin | | >100 | <0.1 | Clavulanic acid | | 0.5 | <0.1 |
| Cefotetan | | — | <0.1 | | | | |

N: Not inhibited, —: undetermined.

Table 3. Effects of inhibitors and ions on the activity of β-lactamase from *Klebsiella oxytoca* GN10650.

| Inhibitor or ion | Concentration (mM) | Inhibition (%) |
|---|---|---|
| *p*-CMB | 0.25 | 91.2 |
| Iodine | 0.1 | 100.0 |
| EDTA | 3.0 | 5.0 |
| $CuSO_4$ | 1.0 | 20.0 |
| $FeSO_4$ | 1.0 | 87.2 |
| $MgSO_4$ | 1.0 | 0 |
| $ZnSO_4$ | 1.0 | 33.6 |
| Clavulanic acid | 0.1 | 100.0 |

Inhibitors were preincubated with the enzyme for 10 minutes at 30°C before addition of the substrate (100 μM cephaloridine).

Table 4. Neutralization of β-lactamase activity by the antibody against β-lactamase from *Klebsiella oxytoca* GN10650.

| β-Lactamase from | Neutralization (%) |
|---|---|
| *K. oxytoca* GN10650 | 100 |
| *K. oxytoca* GN10553 | 94 |
| *K. oxytoca* GN10639 | 96 |
| *K. oxytoca* GN10641 | 97 |
| *K. oxytoca* GN10647 | 94 |
| *K. pneumoniae* GN69 | 0 |
| *Citrobacter freundii* GN346 | 0 |
| *Enterobacter cloacae* GN76 | 0 |
| *Proteus vulgaris* GN7919 | 0 |
| *P. rettgeri* GN4430 | 0 |
| *Morganella morganii* GN5407 | 0 |
| *Pseudomonas cepacia* GN11164 | 0 |
| *P. aeruginosa* GN10362 | 0 |
| *Xanthomonas maltophilia* GN12873 | 0 |
| *Serratia marcescens* GN10857 | 0 |

Neutralization of β-lactamase activity was expressed by the following equation; (a−b)/a (%), where a is enzyme activity without antiserum, and b is enzyme activity with antiserum.

phenol/sulfuric acid method[15].

## Enzymatic Properties of the Purified Enzyme

The kinetic parameters of the enzyme of strain GN10650 (*Km*, relative Vmax, and *Ki*) for various substrates were determined for the enzyme of strain GN10650. The enzyme showed hydrolyzing activity against benzylpenicillin, ampicillin, cephaloridine, cephalothin, cefamandole, carbenicillin, cefuroxime, cloxacillin, aztreonam, cefsulodin, cephalexin, and cefotaxime but not against the cephamycins tested (Table 2). The enzyme was most active at pH 7.0 and its temperature optimum was 50°C under the condition of assay. The effects of some inhibitors and ions on enzyme activity are shown in Table 3. The enzyme activity was almost completely inhibited by iodine, clavulanic acid, *p*-chloromercuribenzoate (*p*-CMB), and $Fe^{++}$ but not by $Mg^{++}$, $Mn^{++}$, and EDTA.

## Immunological Properties of the Purified Enzyme

The effect of anti-GN10650 rabbit serum on the purified β-lactamase was studied using the methods

described by JACK and RICHMOND[16]. A standard curve was obtained when increasing quantities of the antibody were added to a fixed quantity of purified enzyme (constant antigen titration). The enzyme activity was completely inhibited even by a 64-fold dilution of anti-GN10650 rabbit serum (data not shown).

The neutralizing activity of the anti-GN10650 β-lactamase antibody was examined also for various β-lactamases obtained from clinical isolates including five strains of *K. oxytoca* and one strain of each of *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Providencia rettgeri*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Xanthomonas maltophilia*, and *Serratia marcescens* (Table 4), and with four types of plasmid-mediated penicillinase (data not shown). The antiserum completely neutralized (94 to 100%) the β-lactamase from the *K. oxytoca* strains but the other β-lactamases were not affected.

## Discussion

Our previous studies showed that chromosomal β-lactamases from most Gram-negative rods hydrolyze cephalosporins more rapidly than penicillins. These enzymes are called cephalosporinases[17]. In the present study, a β-lactamase purified from *K. oxytoca* GN10650 exhibited properties different from those of the typical cephalosporinases described by SAWAI *et al.*[14], which correspond to the "Class I" β-lactamases described by RICHMOND and SYKES[18].

As indicated by Vmax values, the *K. oxytoca* enzyme could hydrolyze cefuroxime and aztreonam; both are known to be resistant to hydrolysis by cephalosporinase-type β-lactamses[17]. Studies in our laboratory have proved that oxime-type cephalosporins such as cefuroxime, cefotaxime, and ceftizoxime were easily hydrolyzed by β-lactamases from *B. fragilis* GN11477[19], *P. vulgaris* GN7919[20], *P. cepacia* GN11164[21], and L-2 but not L-1 from *X. maltophilia* GN12873[22]. The activities of these enzymes were inhibited by clavulanic acid, sulbactam, imipenem, iodine and *p*-CMB but not by EDTA. Cephamycin derivatives also inhibited the activity of β-lactamases from *B. fragilis* GN11477, *P. vulgaris* GN7919, and *X. maltophilia* GN12873 but not from *P. cepacia* GN11164.

With respect to broad substrate specificity, isoelectric point, and sensitivity of inhibitors, the enzyme from *K. oxytoca* GN10650 resembles the β-lactamase produced by *P. cepacia* GN11164. However the enzyme of *K. oxytoca* is an acidic protein with a pI of 5.3 whereas the pI of the *P. cepacia* enzyme is 9.3[21]. The enzyme of *K. oxytoca* did not cross react with any chromosomal enzyme tested. We, therefore, consider the β-lactamase from *K. oxytoca* GN10650 to be a species-specific cephalosporinase.

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